ARS FUSARIUM/FUMONISIN WORKSHOP

Beltsville, Maryland

September 25-26, 1995

Agricultural Research Service

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ARS Workshop on Fusarium Toxins

September 25-26, 1995
Building 005, Room 21
Beltsville, Maryland

ARS LABORATORIES CONDUCTING RESEARCH WITH FUSARIUM SPECIES AND THEIR TOXINS

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ARS FUSARIUM/FUMONISIN WORKSHOP

Fumonisins burst into world food safety concern in 1988 when the South African group (Marasas, Gelderbloom and associates) identified fumonisin B1 (FB1) as a toxic metabolite produced by *Fusarium moniliforme*, demonstrated that FB1 produced leukoencephalomalacia in a horse, and proposed a possible link of *F. moniliforme* infected corn to esophageal cancer in humans. (The fungal metabolites moniliformin and fusarin C had been found previously in corn but were known not to be the cause of equine leucoencephalomalacia). Doubtless the identity of fumonisins was long delayed because it is a water soluble compound and most toxic fungal metabolites with which scientists have had experience are fat soluble.

Since 1988, research with fumonisins has proceeded at a swift pace. We know far more about fumonisins, chemically and biologically, than was known about aflatoxins seven years after establishment of their chemical structure in 1965. This can be attributed to (1) the rapid development of scientific technologies that has taken place in the last 30 years, and (2) to the recognition by agricultural and other researchers that fungi do produce toxic secondary metabolites which can appear in enormous quantities in food and feed commodities in years when environmental conditions are favorable for their production.

We are fortunate that aflatoxins came first and sensitized the research community, since the control of fumonisins is more complex and challenging than even that of the aflatoxins. Fumonisins are water soluble and do not carry a built in fluorescent tag to simplify detection as do aflatoxins. *Fusarium moniliforme* produces many more toxins than does *Aspergillus flavus* and *A. parasiticus*, and *F. moniliforme* has a sexual phase of reproduction although this may actually confer advantages in study of its biology. As with the aflatoxins, we do not yet know the significance of these secondary metabolites to the fungus or to the host plant and, with the possible exception of fusaric acid, we know little of the effects of *F. moniliforme* toxins to animals beyond that of the fumonisins. Lastly, and probably most important, *Fusarium moniliforme* is a true endophyte of corn. It can be transmitted through the seed to succeeding crop generations and, like many endophytes, its presence may actually afford some advantages to the host corn plant. This relationship may complicate the development of control strategies.

ARS has a major Fusarium/fumonisin research program. Research accomplishments by scientists working on fumonisins are an excellent example of the strength of ARS research. ARS initiated research into the biological effects of *Fusarium moniliforme* 3 years prior to the discovery of the fumonisins. Since 1988 ARS scientists have made many important contributions to the state of knowledge about the biosynthesis, distribution, toxicity and mode of action of this widespread and important mycotoxin. Much of the rapid progress in this research area is due to the ability of ARS scientists to form multidisciplinary teams within and among ARS locations to work together to solve critical problems in agriculture. Equally important are the many collaborations with researchers from other agencies, universities, and industry.

ARS scientists at the Athens location were the first to find fumonisin B_1 in naturally contaminated corn. Surveys of isolates of *F. moniliforme* mating population A by ARS scientists in Peoria found that most strains could produce large amounts of fumonisins when grown on corn in the laboratory. ARS scientists developed methods to measure fumonisins in corn and corn products and freely distributed analytical reference samples of fumonisin B_1 to other scientists world wide at a time when it was simply not available from any other source. In the fall of 1989 and winter of 1990 a large number of disease problems occurred with animals who had eaten corn screenings from the 1989 corn crop in the Midwest and Texas. These included a large number of cases of Equine leukoencephalomalacia (ELEM), a devastating disease in horses, and cases of porcine pulmonary edema. Scientists in Peoria and at the USDA-APHIS National Veterinary Services Laboratories and their collaborators found high levels of

fumonisins associated with these disease outbreaks. These data formed the framework of current advisory levels of concern set for fumonisin exposure levels in farm animals.

Noting the similarity of the structure of fumonisins to sphingolipids, ARS scientists at the Athens location in collaboration with Emory University scientists developed and tested the now widely accepted hypothesis on the mode of action of fumonisins. They found that fumonisins inhibit a step in the sphingolipid biosynthesis pathway in plants and animals, and the toxicity of fumonisins is closely correlated with disruption of sphingolipid metabolism. Sphingolipids are now recognized as important signal molecules in many biological pathways, including cell proliferation and cell death. Toxicology studies by ARS scientists at Athens confirmed the toxicity of fumonisins to rat liver reported by the South Africans and identified the kidney as a major target of fumonisins. ARS scientists at Peoria prepared deuterium, tritium, ^{13}C and ^{14}C labeled fumonisin B_1. The stable isotopes were used as analytical standards for mass spectrometry based quantitative methods, while the radio labeled fumonisins were used in pharmacokinetic studies at the ARS Athens location. These studies showed that the bulk of fumonisins are very rapidly excreted with small levels retained in kidney and liver, the target organs.

ARS scientists have also had key roles planning and implementing a major 1-year FDA-USDA/ARS-National Institutes of Environmental Health Sciences/National Toxicology Program (NIEHS/NTP) collaborative toxicology study of fumonisin B_1 designed to provide data needed by regulators to make risk assessments for human exposure to fumonisins. Scientists at the Athens location did all the preliminary studies that were used to set dose levels for the ongoing study. Commercially purchased, the 1000 grams of fumonisin B_1 needed for this study would have cost in excess of 25 million dollars. ARS scientists in Peoria developed the methods of large-scale purification and trained the scientist at FDA who purified the fumonisin B_1 and collaborated in evaluation of the purified product. ARS scientists in collaboration with FDA scientists at the National Center for Toxicology Research (NCTR) and scientists from Agriculture Canada developed a reference standard batch of fumonisin B_1 for use in comparing the purity of fumonisins used in toxicology studies worldwide and developed criteria for determining the purity of fumonisin B_1 used in the FDA/ARS/NTP study.

Another Fusarium toxin of food safety importance is vomitoxin, produced by *Fusarium graminearum* in wheat and barley. In developing control strategies, ARS scientists at Peoria obtained the first APHIS approval for field testing of a transformation mutant of the pathogenic fungus, *Fusarium graminearum*. In these studies, trichothecene non-producing mutants of *F. graminearum* produced by transformation mediated gene disruption caused less disease and toxin production than that from the wild type trichothecene producing strain from which they were derived.

Future ARS research to reduce Fusarium toxins in corn and wheat will utilize several strategies. First, ARS will seek to understand the role of fumonisins in plant/fungal interactions to form a firm basis for optimizing control strategies to increase plant resistance and reduce the occurrance of fumonisins in corn. These control strategies will utilize genetic approaches involving the introduction of antifungal proteins to control fungal growth and toxin production, and biocontrol procedures to prevent both *Fusarium moniliforme* growth and fumonisin accumulation in corn and *Fusarium graminearum* growth and vomitoxin accumulation in wheat. In vitro screening assays, as well as chemical analyses, used to evaluate new processing changes or preconditioning of grains can reduce or eliminate mycotoxins from processed grain products.

The purpose of this ARS Fusarium/Fumonisin Workshop was to bring together the ARS scientists and others working to solve this agricultural and public health problem in order to review research progress, to facilitate communication among the scientists, and to provide a forum for generating new research ideas. The following abstracts of presentations made at the workshop document the ongoing ARS research on Fusarium toxins and, in particular, fumonisin..

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ABSTRACTS OF PRESENTATIONS

INTRODUCTION

A WORLD WIDE WEB OF MYCOTOXIN RESEARCH--THE *FUSARIUM* CONNECTION

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Often we in scientific research get bogged down in our own investigative activities and sometimes it may appear that we are working alone or only within our specific small group where team investigations occur. This is often the same picture that we paint for our administrators who see us escape from our laboratories for an occasional scientific meeting; the benefits of the latter to U.S. agriculture often far exceed the expense of having us attend. Our collaborations with other research institutions, universities, agribusinesses, and commodity groups are often very informal and there is little administrative record of this activity. I'd like to present a brief look at the overall activity of *Fusarium* research of three major ARS laboratories insofar as they are in touch with what is happening in this area of endeavor on a world wide scale. These three laboratories are the Richard Russell Research Center in Athens, GA, the Food Animal Protection Research Laboratory in College Station, TX and the National Center for Agricultural Utilization Research in Peoria, IL. The ARS scientists involved in *Fusarium* research at these three laboratories know what is happening around the world in this area of research activity. Not only is there collaboration among the scientists from these three laboratories but there is common collaboration with other governmental agencies and major universities throughout the United States. Almost all of the major national and international meetings are attended, often as invited speakers, by the scientists engaged in *Fusarium* research at these three laboratories. Because of the close association by the scientists from these laboratories through interaction at meetings, review of publications among the scientists, interaction with their NPL and direct communication among the scientists, there is little chance for a duplicative effort of research activity. The international collaborations among the scientists doing *Fusarium* research at these three laboratories are very extensive and when we look at these activities along with the collaborative research in the United States there is an extremely vast network of collaborative activity among and by these three laboratories. This collaborative effort not only maintains these laboratories at the forefront of this investigative area, where they are primary leaders of such activity, but the most benefit for the dollars expended for research is being attained. Even in these days of constraints of a flat or decreasing research budget, these scientists are the ones who are keeping this ship we call ARS moving forward and staying on the course laid out by our objectives that we established through interaction with our NPL. If funds in support of this research activity were to increase we could raise our sails to catch this windfall and move the ARS ship forward at an even greater speed. In this workshop we will hear portions of some of the work being done by these scientists and I'm sure you will be impressed with the amount and quality of research that is going on and the progress being made in solving problems for the farmer, the entire agribusiness community, including export marketing, **and** the American consumer if not the consumer from all over the world. I congratulate all of them.

TOXICITY TO DOMESTIC ANIMALS

Influence of Fumonisin B_1, Present in *Fusarium moniliforme* Culture Material, and T-2 Toxin on Turkey Poults[1]

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ABSTRACT Diets containing 300 mg fumonisin B_1 (FB_1)/kg of feed and 5 mg T-2 toxin/kg of feed singly or in combination were fed to female turkey poults (Nicholas Large White) from day of hatch to 21 d of age. When compared with controls, 21-d body weight gains were reduced 21% by FB_1, 26% by T-2, and 47% by the combination. The efficiency of feed utilization was adversely affected by FB_1 and the combination of FB_1 and T-2. Relative weights (grams/100 g BW) of the liver and gizzard were increased in poults fed the FB_1 and the combination diets; whereas, the relative weight of the pancreas was increased in all treated groups. All poults were scored for oral lesions using a scale of 1 to 4 (1 = no visible lesions, 4 = severe lesions). Oral lesions were present in all poults fed the T-2 diet (average score of 3.29) or the combination diet (average score of 3.54). Serum concentration of cholesterol was decreased and lactate dehydrogenase activity was increased in poults fed the FB_1 and combination diets. The activity of aspartate aminotransferase and the values for red blood cells, hemoglobin, and hematocrit were increased only in poults fed the combination diet. Inorganic phosphorus concentration was decreased only in poults fed the combination diet. The increased toxicity in poults fed the combination diet for most variables can best be described as additive, although some variables not altered by FB_1 or T-2 singly were significantly affected by the combination, indicating that the combination may pose a potentially greater problem to the turkey industry than either of the mycotoxins individually.
(*Key words*: Fumonisin B_1, T-2 toxin, *Fusarium moniliforme*, toxicity, turkey poult)

ENVIRONMENT AND HEALTH

Effects of Feeding Fumonisin B_1 Present in *Fusarium moniliforme* Culture Material and Aflatoxin Singly and in Combination to Turkey Poults[1]

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ABSTRACT Diets containing 200 mg fumonisin B_1/kg of feed and .75 mg aflatoxin/kg of feed singly or in combination were fed to female turkey poults (Nicholas Large White) from day of hatch to 21 d of age. When compared with controls, 21-d body weight gains were reduced 10% by fumonisin B_1, 39% by aflatoxins, and 47% by the combination. Relative weights (grams/100 g body weight) of the kidney and pancreas increased in poults fed the diet containing aflatoxins alone, whereas the relative weight of the liver decreased. Relative weights of the liver and pancreas increased in the poults fed the fumonisin diet. Relative weights of the kidney, pancreas, and gizzard increased in the poults fed the combination diet, whereas the relative weight of the liver decreased. Most serum constituents, hematology values, and activities of enzymes measured were altered in poults receiving the diets containing aflatoxins with or without fumonisin B_1. No major histological lesions were observed in tissues from control poults or poults fed the diet containing fumonisin alone. Lesions associated with aflatoxins were only observed in the liver and occasionally in the kidney of poults fed the diets containing aflatoxins with or without fumonisin B_1. The primary hepatic change was bile duct hyperplasia with some hepatocellular degeneration and necrosis and megalocytosis. Occasional necrotic and degenerating tubular epithelial cells were observed in the kidneys. The increased toxicity in poults fed the combination diet for most variables can best be described as additive, although some variables showed less than additive toxicity. Although toxicity due to fumonisin B_1 was shown, it must be pointed out that these concentrations are not likely to be encountered under field conditions. However, other stress factors may be present that could alter the toxicity observed under these controlled experimental conditions.
(*Key words*: Fumonisin B_1, aflatoxins, *Fusarium moniliforme*, toxicity, turkey)

Acute Hepatic and Renal Toxicity in Lambs Dosed with Fumonisin-Containing Culture Material[1]

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ABSTRACT: To examine the effects of acute exposure to fumonisin-containing culture material (FCCM), 15 crossbred wether lambs were dosed intraruminally with FCCM containing 0 (CONTROL, n = 3), 11.1 (LOW, n = 4), 22.2 (MED, n = 4), or 45.5 (HIGH, n = 4) mg of total fumonisins (B_1, B_2, and B_3)/kg BW daily for 4 d. Blood samples were collected daily, and on d 11 lambs were killed and necropsied. Changes in serum constituents in fumonisin-treated lambs indicative of liver damage, included increased ($P < .05$) activities of alkaline phosphatase, γ-glutamyl transferase, aspartate aminotransferase, and lactate dehydrogenase. Serum concentrations of cholesterol, triglycerides, urea nitrogen, and creatinine were also increased ($P < .05$) in lambs dosed with FCCM. Hemoglobin tended to increase ($P = .07$) and white blood cell count tended to decrease ($P = .08$) in HIGH lambs and activated partial thromboplastin time tended to decrease ($P < .10$) in lambs dosed with LOW and MED treatments. Mitogen-induced lymphocyte blastogenesis was not different ($P = .14$) among treatments. Feed intake markedly decreased ($P < .01$) following the first dosing of FCCM and continued to decline throughout the study. Ruminal VFA concentrations and pH tended to decrease ($P < .10$) at d 11 in treated lambs. Relative liver and kidney weights (g/100 g of BW) increased ($P < .05$) in fumonisin-treated lambs. Histological examination revealed tubular nephrosis and mild hepatopathy in dosed lambs. Lambs receiving the HIGH treatment died on d 3, 4, 5, and 7 of the study and on d 9 one lamb on the MED treatment died. Diarrhea and lethargy were observed in fumonisin-treated lambs. Fumonisin-containing culture material dosed orally is acutely toxic to sheep, affecting primarily liver and kidney function.

Interactive Toxicity of Fumonisin B_1 and Deoxynivalenol-Contaminated Diets Fed in Combination to Growing Swine

R.B. HARVEY, T.S. EDRINGTON, L.F. KUBENA

The effects of fumonisin B_1 (FB_1)-contaminated diets (from *Fusarium moniliforme* cultural material) and deoxynivalenol (DON)-contaminated (from naturally contaminated wheat) diets, fed singly and in combination to growing barrows were evaluated. Six barrows (3 replicates of 2 each, mean body weight, 13.6 kg) per group were fed: 0 mg FB_1, 0 mg DON/kg feed; 100 mg FB_1/kg feed; 5 mg DON/kg feed; or 100 mg FB_1 plus 5 mg DON/kg feed for 28 days. Body weight or weight gain was not affected by either toxin singly; however, the toxins in combination significantly ($P<0.01$) decreased these measurements in a synergistic fashion. Feed consumption was reduced by the combination treatment but not by single toxin treatments. Serum biochemical analytes were adversely affected by FB_1 and by the FB_1 plus DON treatments, whereas DON alone did not affect these measurements. Hematologic values were affected by the FB_1 plus DON and cell-mediated immune response was decreased by FB_1 and further reduced by the FB_1 plus DON treatment. Liver weight was increased by the FB_1 treatment, yet was decreased in a highly significant manner by the combination toxin treatment. For the parameters we evaluated, measurements were affected more by the combination of these two *Fusarium* toxins than either toxin alone and the interactions could be described as more than additive and often times were synergistic.

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BIOLOLGICAL ACTIVITY IN LABORATORY ANIMALS AND IN VITRO SYSTEMS

COMPARATIVE SUBCHRONIC TOXICITY STUDIES OF NIXTAMALIZED AND WATER-EXTRACTED *FUSARIUM MONILIFORME* CULTURE MATERIAL. KA Voss, CW Bacon, FI Meredith, & WP Norred. Toxicology & Mycotoxin Research Unit, ARS, USDA, Athens, GA.

Fumonisins are mycotoxins produced by *Fusarium moniliforme*, *F. proliferatum*, and other *Fusarium* species which are found on corn and other cereal grains. Fumonisins cause a variety of species-specific toxicoses and have been linked to human esophageal cancer in areas of southern Africa and China where corn is a dietary staple. Nixtamalization is the process by which masa flour is made from corn. During nixtamalization, which is essentially a base hydrolysis, fumonisins are converted to their hydrolyzed forms. To study the effect of nixtamalization on the organ-specific toxicity of *F. moniliforme*-containing culture material (CM) as well as to compare the effectiveness of nixtamalization and water extraction for detoxification of the CM, male rats (n=10/group) were fed diets containing equivalent weights of CM, nixtamalized CM (NX), or water extracted CM (WE) for four weeks. An additional control group was fed a diet containing sound corn (SC). The NX diet contained 58 ppm hydrolyzed fumonisin B1 (HFB1), but no detectable fumonisin B1 (FB1). Water extraction removed most of the FB1 from the CM and, as a result, the WE and CM diets contained 8 and 71 ppm FB1, respectively. Body weight gain of all four groups differed significantly ($p<0.05$) in the order: SC>WE>NX>CM. Serum chemical, organ-weight, and histopathological findings typical of FB1-induced hepatopathy and nephropathy was found in both the CM and NX groups. In contrast to renal lesions, which were of similar severity in each of these groups, liver lesions found in the NX group were less extensive than those of CM fed rats. Hepatopathy was not found in rats fed the WE diet. However, kidney lesions indistinguishable from those found in the CM and NX groups were found in all rats from the WE group and could be attributed to the residual FB1 remaining in the CM following water extraction. Thus, the organ-specific effects of nixtamalized CM containing HFB1 were similar to those of the FB1-containing CM and nixtamalization was not as effective as water extraction for the detoxifying the CM. The data further suggest that, in rats, the hepato- and nephrotoxic effects of HFB1 and FB1 are similar.

FERTILITY AND REPRODUCTIVE PERFORMANCE IN RATS FED FUMONISIN B1 CONTAINING CULTURE MATERIAL OF *FUSARIUM MONILIFORME* STRAIN MRC 826.

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Fumonisins are mycotoxins produced by *Fusarium moniliforme* and other *Fusarium* species. They are commonly found in corn and corn-based feeds and foodstuffs. Fumonisins disrupt sphingolipid (SL) metabolism and alter cellular SL profiles which in turn may alter cell proliferation and differentiation, both of which are important in reproduction. To study the effect of *F. moniliforme* on fertility and reproductive performance, diets formulated with culture material of toxic *F. moniliforme* strain MRC 826 (CM) to provide 0, 1, 10 or 55 ppm fumonisin B1 (FB1) were fed to male and female rats beginning 9 and 2 weeks, respectively, before mating. and continuing throughout the mating, gestational, and lactational phases of the study. CM caused nephropathy typical of FB1 in males fed $\geq$ 10 ppm FB1 and females fed 55 ppm FB1. No significant reproductive effects were found in males (n=12/group), dams and fetuses examined on gestation day 15 (G15, n$\geq$ 8/group), or dams and litters through day 21 *post partum* (n$\geq$9/group). Litter weight gain in the 10 and 55 ppm FB1groups was slightly decreased; however, gross litter weight and physical development of the offspring were not affected. Altered SL ratios indicative fumonisin exposure, specifically increased sphinganine to sphingosine ratios, were found in the liver of dams from the 55 ppm group on G15. In contrast, SL ratios of abdominal slices, containing liver and kidney, of G15 fetuses from the control and high-dose litters did not differ. In a second experiment, two dams were injected intravenously on G15 with 101 µg [14C]FB1 (3.179×10^5 dpm). After one hour, which allowed for ca. 98% of the dose to be cleared from the maternal blood, measurable amounts of radioactivity were not found in the fetuses. These results indicate that the CM, and by inference FB1, did not have significant reproductive effects at doses which were minimally toxic to the dams and, based upon two lines of evidence, further suggest that negligible *in utero* FB1 exposure occurred through G15.

USE OF TISSUE SLICES AS A SCREENING TOOL FOR FUMONISIN-LIKE ACTIVITY

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The fungus, *Fusarium moniliforme*, commonly contaminates corn, and produces the fumonisin mycotoxins. These toxins are the responsible agents of field intoxications in animals, including equine leucoencephalomalacia and porcine pulmonary edema. Fumonisins also cause liver and kidney toxicity in laboratory rodents, are tumor promoters, and may have cancer-initiating ability. The mode of action of the fumonisins is thought to be their ability to inhibit ceramide synthase, which catalyzes the conversion of sphinganine (Sa) and sphingosine (So) to ceramide. The result of this inhibition is disruption of sphingolipid biosynthesis, and accumulation of sphingoid bases, primarily Sa, in biological fluids and tissues. This property of fumonisins was utilized to develop a bioassay system that can be used to detect elevated free sphingoid bases, and thereby fumonisin-like activity. Precision-cut tissue slices were prepared from fresh cores of liver or kidney, maintained in media in an incubator, and exposed to fumonisin or test materials. After various periods of time the slices were removed, and analyzed for content of Sa and So. Slices exposed to fumonisins for short (12 to 48 hr) periods were found to have greatly elevated free Sa content. We also studied the specificity of the response by comparing hepatotoxins that act by differing mechanisms for their ability to elevate intracellular Sa. These toxins included aflatoxin B_1, beauvericin, carbon tetrachloride, cyclopiazonic acid, carbonyl cyanide, and fumonisin B_1. Slices were exposed to the toxins or exogenous Sa for 20 hrs, and then analyzed for intracellular free Sa content. Only fumonisin B_1 (FB_1) and exogenous Sa significantly elevated intracellular Sa. In control slices and slices dosed with toxins other than FB_1, free Sa content was <1.5 nmol/g tissue. Slices dosed with FB_1 or exogenous Sa had 5-15 nmol/g tissue. The utility of the bioassay was determined by investigating whether acetonitrile:water (1:1) extracts of corn naturally contaminated with fumonisins could elevate Sa in rat liver slices. The extracts significantly elevated Sa in the slices, even when dosed with extract equivalent to as little as 0.5 mg of corn. We have also utilized the bioassay to evaluate a potential decontamination procedure for fumonisin-contaminated corn developed by Texas A&M researchers. Although the method (ozonolysis) destroys fumonisin B_1 in aqueous solution, as determined by HPLC analysis, the by-products of ozonolysis are likely to be toxic, since they can inhibit ceramide synthase. Other proposed uses for the tissue slice bioassay include screening for fumonisin-like activity in cultures of fungi other than *F. moniliforme*, and evaluating the fumonisin-like activity of feeds associated with field problems of unknown etiology.

EFFECTS OF FUMONISINS AND SPHINGOLIPIDS ON TURKEY LYMPHOCYTES AND A HUMAN ASTROCYTOMA CELL LINE

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Fusarium moniliforme and *F. proliferatum*, fungi frequently found as contaminants on corn, sorghum and other grains, are capable of producing fumonisins. Mortality and serological, hematological and pathological effects have occurred in broiler chicks fed *F. proliferatum* culture material containing known concentrations of fumonisin, moniliformin and beauvericin. In vitro assays were performed to determine the effects of these toxins. When turkey lymphocytes were incubated for 72 hours with 8 μM fumonisin B_1 or B_2 or 50 μM beauvericin, internucleosomal DNA fragmentation and morphological features characteristic of apoptosis were observed. Fumonisin B_1 (IC_{50} = 1.9 μM), fumonisin B_2 (IC_{50} = 0.5 μM) and hydrolyzed fumonisin B_1 (IC_{50} = 10 μM), but not moniliformin, caused inhibition of proliferation. To study the role of fumonisins as inhibitors of ceramide synthesis, on inflammatory reactions in the central nervous system, the effects of sphingosine, C2-ceramide and fumonisin B_1 on cytokine (IL-1β)-induced IL-8 production in a human astrocytoma cell line (U373MG) were determined. Sphingosine, C2-ceramide and fumonisin B_1 each enhanced IL-8 production, but they partially suppressed IL-1β-induced DNA synthesis. In the absence of cytokine, sphingosine, but not fumonisin B_1, stimulated the cells to proliferate. Metallothionein was reduced in the presence of fumonisin B_1. By elucidating biomechanisms involving *Fusarium* metabolites, the potential for toxic effects at low, environmental doses will be understood and means of detoxification will be identified.

Lactational Passage of Fusaric Acid from the Feed of Nursing Dams to the Neonate Rat and Effects on Pineal Neurochemistry in the F1 and F2 Generations at Weaning

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Fusaric acid is produced by several *Fusarium* species and found in corn, corn based foods and feeds, wheat, barley, and other cereal grains. Given parenterally to rats, the mycotoxin affects neurochemical parameters in the pineal gland associated with growth and maturation. Since little information exist concerning the oral effects of fusaric acid, the mycotoxin was mixed in diets at 10, 75, and 200 ppm and fed to pregnant rats (F0-dams) beginning the third week of gestation, through parturition, and weaning (F1 generation). On day 4 postpartum, F1-pups were culled to 9-10 pups/litter, the stomach colostrum collected from the culls and analyzed for fusaric acid (GC/MS). The mycotoxin in the colostrum (ng fusaric acid/100 mg colostrum) was directly related to the amount consumed by the nursing dams (i.e., 200 ppm pups=3547 ng; 75 ppm pups=1449 ng; 10 ppm pups=80 ng; controls pups=18 ng). All other animals survived, appeared normal, healthy, and in good pelage. F0-dam feed consumption and dam and pup weights were not statistically different, but there was an inverse relation between pup average weight gain and amount of fusaric acid in the diets (i.e., weight gains: control pup>10 ppm pup>75 ppm pups>200 ppm pups). At weaning, the F1-pups were randomly assigned to two groups/treatment: Group one (F1A) for reproduction and fusaric acid effects on the F2 generation; Group two (F1B) for neurochemical comparisons. The F1A rats were maintained on their respective diets to age 13-14 weeks; animals were bred (i.e., controls males x controls females, 10 ppm x 10 ppm, etc.) and the F1A-dams and F2-pups monitored as above. Weight gains and fusaric acid in stomach colostrum from the F2-culls were analogous to the F1 generation. Also, the amount of colostrum collected from the F2 (4-day old) culls was compared. The 75- and 200-ppm pups had 51% and 38%, respectively, less than the controls or the 10 ppm pups (i.e., average amount of colostrum in the stomach/pup/treatment, mg cls/pup: control culls=337.84, n=27; 10 ppm culls=357.78, n=24; 75 ppm culls=167.00, n=19 ; 200 ppm culls=207.89, n=22; $P<0.16$). These values tend to support either decreased milk production by the dams and/or decreased suckling times by the neonates.

On days 5-8 postpartum, using pup average weight gains as an indication of milk production in F1A-dams (i.e., weigh-suckle-weigh method; controls vs 200 ppm), the controls gained 30% more than the 200 ppm F2-pups ($P<0.05$). At weaning, no differences were observed in neurochemicals (HPLC/EC-Flu) in the pineal gland for the F1 generation. However, in the F2-200 ppm male and female weanlings, pineal serotonin and tyrosine were significantly decreased ($P<0.05$). Additionally, both serum and pineal melatonin (ELISA; HPLC/EC) in both sexes were increased by fusaric acid (male F2-200 ppm pups vs controls, 53.46 vs 35.59 pg/ml, $P<0.24$; female F2-200 ppm pups vs controls, 60.0 vs 40.9 pg/ml, $P<0.03$). Prolonged imbalances in pineal tyrosine, serotonin, and melatonin in either sex may adversely affect growth, maturation, and/or reproduction.

The results indicate fusaric acid in diets at $\leq$0.3 ppm (i.e., background control diet) lactationally passes from nursing dams to the neonate; at 200 ppm, fusaric acid decreases pineal serotonin and tyrosine and increases melatonin in both the pineal gland and serum of weanling rats. The data also suggest limited neonate weight gains may be related to either decreased milk production in dams or mycotoxin effects on the suckling neonate.

Fusaric Acid in *Fusarium moniliforme* Cultures, Corn, and Feeds Toxic to Livestock and the Neurochemical Effects in the Brain and Pineal Gland of Rats

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Fusaric acid is produced by several species of *Fusarium*, which commonly infect corn and other agricultural commodities. Since this mycotoxin may augment the effects of other *Fusarium* toxins, a gas chromatography/mass spectrometry method of analysis (GC/MS) in feeds was developed. Fusaric acid was analyzed as the trimethylsilyl-ester from *F. moniliforme*-cultures, *F. moniliforme*-contaminated corn screenings, and feeds toxic to livestock. The mycotoxin was found in all samples and ranged from 0.43 to 12.39 μg/g sample. Also, fusaric acid was tested for its neurochemical effects in the brain and pineal gland of rats. Animals were dosed intraperitoneally (100 mg/kg body weight) 30 minutes prior to the onset of the dark phase (lights out) and the effects were studied at 1.5, 3.5, 5.5 hours after treatment. As measured by high-performance liquid chromatography with electrochemical and fluorescent detections (HPLC/EC-Flu), brain serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA), tyrosine (TYRO), and dopamine (DA) were increased ($P<0.05$) by fusaric acid, while brain norepinephrine (NEpi) was decreased ($P<0.05$). Analogously, DA in the pineal gland increased and NEpi decreased ($P<0.05$). Pineal N-acetylserotonin (NAc5HT) was increased ($P<0.05$), whereas pineal 5HT and its two major metabolites 5HIAA and 5-hydroxytrytophol (5HTOL) decreased ($P<0.05$). Elevated brain TYRO and brain and pineal DA, with decreased NEpi, may be consistent with fusaric acid's partial inhibitory effect on tyrosine-hydroxylase and its inhibitory effect on dopamine-β-hydroxylase, respectively. Elevated pineal NAc5HT is consistent with decreased pineal 5HT and the increased pineal DA, and support the dopaminergic stimulatory activity of the enzyme responsible for the conversion of 5HT to NAc5HT. This is the first report of fusaric acid's *in vivo* effect on pineal DA, NEpi, 5HT, and NAc5HT in rats, and a relation for the effects on TYRO, 5HT, and 5HIAA in brain tissue. The results indicate fusaric acid alters brain and pineal neurotransmitters and may contribute to the toxic effects of *Fusarium*-contaminated feeds.

Effects of *Fusarium* Toxins on Melatonin in Pineal Tissue Cultures: Preliminary Investigations

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Food safety, animal health and productivity, and human health problems identified with fungal-contaminated grains most recently have concentrated research on *Fusarium* species and their toxic metabolites. Major worldwide agricultural problems with *Fusarium* have been recognized since: **a.)** *F. moniliforme* and the fumonisins have been associated with equine leukoencephalomalacia; **b.)** the human oncologic implications with esophageal cancer in certain areas of South Africa, China, and possibly Iran; and **c.)** the routine occurrence of the fumonisins and other *Fusarium* toxins (i.e., fusaric acid, vomitoxin, tricothecenes, zearalenone) in corn, wheat, barley, rice, sorghum, rye, millet, and oats.

Regulation governing the amount of mycotoxins found in agricultural commodities used for both animal and human consumption are generally based on toxicologic investigations with pure compounds. Currently, the combined effects of *Fusarium* toxins on animal and human health have caused concerns because synergistic activities present a unique set of problems with defining both toxicity and food safety guidelines.

The effects of fusaric acid and fumonisin B1in the fertile chicken egg is inherently more toxic than the individual mycotoxins. Furthermore, fusaric acid lactationally passes from the feed of nursing dams to the neonate rat; the mycotoxin either decreases milk production in dams on a diet containing fusaric acid or affects neonate capabilities to use milk; also the mycotoxin alters tyrosine, serotonin, and melatonin in the pineal gland of the offspring at weaning. The pineal gland, with associated neurotransmitters, has been defined as the neurotransducer most responsible for seasonal physiological and endocrine changes in both animals and humans. Imbalances in melatonin, and/or the neurotransmitters associated with melatonin production may adversely affect growth, maturation, and reproduction in animals consuming feed contaminated with this mycotoxin. Research also suggest fusaric acid synergistically affects the toxicity of other mycotoxins. Therefore, to screen the combined effects of *Fusarium* toxins on melatonin as they occur in infected foods and feeds, rat pineal tissue cultures and an enzyme linked immunosorbent assay (ELISA) for melatonin were developed.

Fusaric acid at 1μM and 100 μM increased pineal tissue culture melatonin vs controls in a dose dependent manner (i.e., 103% and 301%, respectively); *in vitro* melatonin increases by fusaric acid are consistent with the *in vivo* effects (orally) of the mycotoxin in the serum and pineal gland of weanling rats. Beauverisin, another *Fusarium* toxin, at 1μM increased pineal tissue culture melatonin vs controls 33%. Fusaric acid and beauverisin together at 1μM each increased melatonin 310%; an effect comparable to 100 μM fusaric acid. These results, along with previous investigations, support the hypothesis that fusaric acid in combination with other mycotoxins is more toxic than the individual compounds. Also, the results substantiate rat pineal tissue cultures may be used as a screening tool for assessing the combined *in vivo* effects of toxins' interaction. *In vivo* and *in vitro* studies are continuing on the combined effects of fusaric acid, fumonisins, vomitoxin, zearalenone, and selected tricothecenes on melatonin and related neurotransmitters, and their assessment to growth, maturation, and behavior in the rat model.

PURIFICATION AND DETECTION OF FUMONISINS

Purification of FB_1 and FB_2 from Rice Culture

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ABSTRACT

Procedures are presented for growing *Fusarium moniliforme* MRC 826 on rice, separation of fumonisin B_1 (FB_1) from fumonisin B_2 (FB_2), purification of FB_1 and preliminary procedures for purification of FB_2.

The mycotoxins were extracted from rice culture material (RCM) with acetonitrile-water (1:1), filtered, and the acetonitrile removed on a rotary evaporator.

Preparative reverse phase liquid chromatography (LC) was used to isolate and partially purify FB_1 and FB_2 from the extract. The extract was applied to a C_{18} reverse phase cartridge. FB_1 and FB_2 were eluted from the cartridge by a gradient of water-acetonitrile at a flow rate of 30 mL/min. A second preparative LC procedure using 0.5% pyridine-water and two CN cartridges was used to purify FB_1.

The FB_2 fraction was concentrated on a rotary evaporator to remove the acetonitrile. Acetonitrile was added back in sufficient quantity to redissolve the crystallin material in the fraction. An aliquot of the FB_2 fraction was added to a centrifugal spinning silicic acid TLC plate. The centrifugal TLC plate was washed at 3 mL/min with a linear gradient of (A) chloroform-acetone(4:3) and (B) methanol-acetone (1:1) to elute the FB_2. Gradient starting conditions were 10% methanol and ending conditions were 50% methanol. This preliminary study using the centrifugal spinning TLC showed the procedure to have the potential to be useful for purification of FB_2.

RAPID PURIFICATION OF FUMONISINS B_3 AND B_4 WITH SOLID PHASE EXTRACTION COLUMNS

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A simple method was developed to isolate fumonisin B_3 and B_4 (FB_3 and FB_4) from cultures of *Fusarium moniliforme*. A novel strain of *F. moniliforme* which only produces FB_3 and FB_4 but no FB_1 or FB_2 was used. Extracts from the culture material are loaded onto NH2 cartridges and the fumonisins eluted with 5% HOAc/MeOH, which was diluted with 1.5 volumes of water and loaded onto a C18 cartridge. The fumonisin were eluted with increasing amounts of CH_3CN in H_2O. Complete separation was obtained between the FB_3 fraction (FB_3 plus about 20% as much FA_3 and a trace of FC_3) and the FB_4 fraction (FB_4 plus about 11 and 18% as much FA_4 and FC_4, respectively). The purities were measure by three methods, fluorescence detection of the OPA derivatives, evaporative light scattering detection of the underivatized fumonisins or by electrospray-MS and the results compared. The fractions were a least 90% fumonisins and probably closer to 95% pure. A 277 mg FB_3 fraction and a 62 mg FB_4 fraction were obtained from the combination of a 10g NH2 and a 10g tC18 cartridge. The method should be useful for providing FB_3 and FB_4 for toxicity studies or for further purification by semi-preparative HPLC. It should also be useful for working with radio-labeled material.

COMPARATIVE ASSESSMENT OF METHODS FOR DETECTING FUMONISINS IN GRAIN BASED FOODS. Chris M. Maragos and Ronald D. Plattner, USDA-ARS-NCAUR, 1815 N. University St., Peoria, IL 61604.

Because of the substantial interest in the fumonisins, a large variety of analytical methods have been reported for quantitation of these mycotoxins. All of the methods utilize an organic extraction of the food, typically using methanol/water or acetonitrile/water mixtures. Prior to detection by most analytical methods the fumonisins are isolated from the organic extract using solid phase extraction columns. In the most widely used procedure the fumonisins are separated by HPLC after reaction with o-phthaldehyde (OPA) to yield a fluorescent derivative. This method has been collaboratively studied. Immunochemical methods that do not require the use of sophisticated instrumentation have also been reported. Several enzyme-linked immunosorbent assays (ELISAs) are commercially available from two sources (Neogen and R-Biopharm). All have cross-reactivity to varying degrees with fumonisins other than fumonisin B_1 (FB_1). None of the commercial kits have been reported to cross-react with the aminopolyol backbone (hydrolyzed fumonisins). The toxicity of the backbone relative to the intact fumonisins is currently under investigation in several laboratories.

Past versions of the ELISA kits, which relied upon the first generation of monoclonal antibodies, would occasionally overestimate fumonisin content by substantial amounts. The latest versions, available only this year, are much more sensitive with limits of detection less than 10 ppb in corn. The increased sensitivity has resulted from the development of newer polyclonal antibodies. The problem of overestimation of fumonisin content has been partially resolved by using the newer kits and by comparing the ELISA results with the sum of the contents of the individual toxins as determined by HPLC. Few of the reports in the literature have used the newer kits, and although several small scale studies are in progress a large scale collaborative study of the newer ELISAs is warranted.

Molecular Modeling Studies of the Fumonisin Mycotoxins

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The fumonisins are mycotoxins that are produced by the corn pathogen, *Fusarium moniliforme*, and have been shown to cause a wide range of toxic effects in numerous animals. They have been shown to have cancer-promoting activity in animals, and have been linked to human esophageal cancer. To date, the only biochemical findings show that the fumonisins are inhibitors of the sphingosine and sphinganine *N*-acyltransferase system. No other biochemical effect of the fumonisins has been established. This paper discusses the molecular models of the lowest energy conformations obtained for fumonisin B_1, B_2, B_3, and B_4 (FB_{1-4}) and the FB_1 backbone. The structure with the lowest energy conformation of FB_1 in relation to iron ligand formation is investigated. The lowest energy conformation of FB_1 based on the stereostructure of FB_1 (SS-FB_1) also is presented. These modeled structures provide insight into why antibodies produced to FB_1 have lower affinities for FB_1 than expected. They also produce insight into other potential activities that may be associated with these molecules.

Development of an Improved Monoclonal Antibody-based ELISA for Fumonisin B_{1-3} and the Use of Molecular Modeling to Explain Observed Detection Limits

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Monoclonal antibodies were prepared against the fumonisins, a group of mycotoxins produced by the plant pathogen, Fusarium moniliforme. *Splenic lymphocytes, from Balb/c mice immunized with fumonisin B_1–ovalbumin conjugate, were fused with SP2/O myeloma cells, and 14 hybridomas were selected. In a competitive enzyme-linked immunosorbent assay, fumonisin B_1–bovine serum albumin and free fumonisin B_1 (FB_1) competed for the monoclonal antibody. The concentrations of FB_1 required to inhibit 50% antibody binding (IC_{50}) ranged from 300 to 670 ppb. Antibodies also cross-reacted with fumonisins B_2 and B_3 (FB_2, FB_3), and the hydrolyzed backbone of fumonisin B_1 (HB-FB_1). None of the 14 monoclonal antibodies recognized the sphingolipids, sphingosine and sphinganine, that are structurally similar to the backbone of the fumonisins. Three-dimensional computer models of FB_1, FB_2 and FB_3 show the amine backbone folding with the two esterified trimethylpropane-1,2,3-tricarboxylic acid side-chains to form a cage into which the hydroxyl and acid groups of these fumonisins extend. The HB-FB_1 molecule, with the two trimethylpropane-1,2,3-tricarboxylic acid esterified moieties at carbons 14 and 15 removed, does not possess two of the three branches which are folded together with inter-hydrogen bonding to formulate the three-dimensional structure that makes up the cage feature of FB_1, FB_2 and FB_3. Because of the unexpected folding of the three arms to make a cage of FB_1, attachment of the protein to the amine group, which is close to, or appears to be part of, the epitope, may have allowed the immune system of the mouse to produce antibodies more specific for the FB_1–protein conjugate than to free FB_1.*

EFFECT OF PROCESSING ON FUSARIUM MYCOTOXINS IN CONTAMINATED CORN

THE EFFECTS OF PROCESSING ON *FUSARIUM* MYCOTOXINS IN CONTAMINATED CORN

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Several frequently occurring mycotoxins have been examined for their distribution in products or fractions resulting from processing of contaminated grains, including: wet milling of corn contaminated with fumonisins. Generally, mycotoxins survive the various processes and can become concentrated in certain fractions causing economic losses because these fractions would normally be used for animal feed. In the present study two lots of naturally contaminated corn containing 1.0 and 13.9 ug/g FB1, respectively, were wet milled, in duplicate, and levels of toxin (FB1 and FB2), determined in the different fractions. No toxin could be found in any fractions, except steep water, from corn having an initial level of 1.0 ug/g FB1. Also,no fumonisins could be detected in the starch fractions (the major mulling product) from the corn originally containing 13.9 ug/g FB1. Other fractions from this corn were contaminated as follows: gluten (5.5 ug/g FB1, 4.8 ug/g FB2) fiber (4.2 ug/g FB1, 2.6 ug/g FB2). A significant portion (22%) of the recoverable fumonisins was found in the steep and process waters. Certain benefits of processing of contaminated grains occur through the production of some mycotoxin-free fractions. Ongoing studies may elucidate process changes or preconditioning of grains which could further reduce or eliminate mycotoxins from processed grain products.

FUSARIUM PLANT INTERACTIONS

MODE OF ACTION OF FUMONISINS AND RELATED COMPOUNDS AND IMPLICATIONS FOR POSSIBLE PLANT CONTROL STRATEGIES. R.T. Riley and C.W. Bacon. Toxicology and Mycotoxin Research Unit, USDA/ARS, Athens, GA 30604-5677.

Recent work in our laboratory has been directed towards elucidation of the biochemical mechanism of action of fumonisins in animal and plants. The results of these studies indicate that fumonisins and the structurally related AAl-toxins are potent and specific inhibitors of de novo sphingolipid biosynthesis (Fig. 1) in animals, plants (including corn), and fungi (yeast). Inhibition of the enzyme ceramide synthase results in disruption of sphingolipid metabolism. Disruption of sphingolipid metabolism results in marked changes in the intracellular concentration of bioactive lipid intermediates and end-products. Many of the affected lipids are known to be involved in intracellular signalling systems which control many aspects of cellular regulation. Proven effects include altered cell-cycle progression, differentiation, programmed cell death, and agonist-induced responses which are coupled to intracellular signal transduction systems. AAl-toxin is a proven pathogenicity factor for *Alternaria alternata* f.sp. *lycopercici*-induced stem canker disease in tomato. Fumonisins have yet to be proven to be pathogenicity or virulence factors in *Fusarium moniliforme*-induced corn diseases. Nonetheless, both groups of toxins inhibit ceramide synthase in susceptible tomato varieties and fumonisins inhibit ceramide synthase in corn. Resistant varieties of tomato also show evidence of disrupted sphingolipid metabolism but appear to be able to metabolism accumulated sphinganine. Sphinganine and its degradation product sphinganine-1-phosphate have been proven to be highly toxic to both animal and plant cells. Thus, the ability of resistant plants to metabolize sphinganine could play a role in resistance to the toxic effects of AAl-toxin and fumonisins in plants. These studies on the mechanism of action have led us to hypothesize that the production of fumonisins by *F. moniliforme* in situ in corn may play an important role in the ecology of the fungal/plant interaction. Understanding the role of fumonisins in the plant/fungal interaction will fill existing gaps in the basic knowledge necessary to develop control strategies aimed at increasing plant resistance and reducing the occurrence of fumonisin in corn.

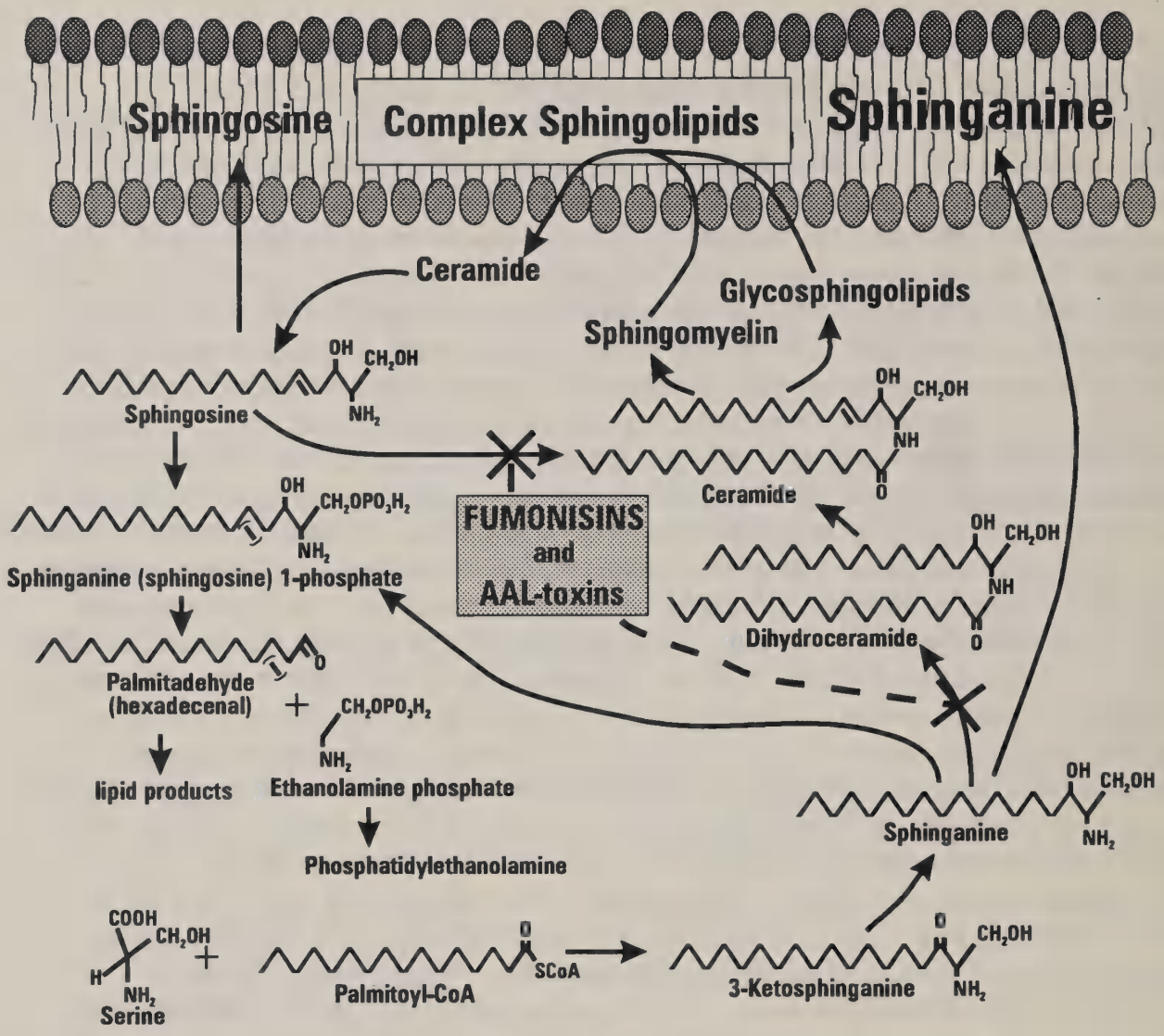

Figure 1. Inhibition of ceramide synthase by FB1 and AAL toxin. The consequences of inhibition are a decrease in de novo sphingosine biosynthesis, a decrease in complex sphingolipids, and an increase in free sphinganine , sphingosine, sphinganine 1--phosphate, phosphatidyl ethanolamine and other phospholipids, lipid products and degradation products.

FUSARIUM MONILIFORME IS A SYMPTOMLESS ENDOPHYTE OF CORN. C.W. Bacon, D.M. Hinton, R.T. Riley, I.E. Yates, and F. Meredith. Toxicology and Mycotoxin Research Unit, USDA, ARS, Athens, GA 30604-5677.

Fusarium moniliforme Sheldon is a nonobligate parasite of corn that produces several mycotoxins. This fungus is also associated with 32 genera of plants. *F. moniliforme* causes systemic infections of corn kernels, which then serve as dissemination vehicles and inoculum sources. Infected plants usually will not show disease symptoms, particularly under well managed systems. A small percent (less than 10%) of all infected plants will develop one of several symptoms of the disease complex associated with infection by this fungus. Symptomless infections were studied at the light and ultrastructural level in corn plants, and was compared with plants that eventually showed symptoms of seedling disease. Three isolates of *F. moniliforme*, a hygromycin mutant of two of these isolates, and *F. fujikuroi* were used to infect surface and internally sterilized corn kernels, and symptomatic and symptomless infections were observed for 8 weeks. The results indicated that in symptomless infected plants, hyphae were intercellular only and distributed throughout the plant, whereas in plants showing disease symptoms, the fungus was both intercellular and intracellular. Early evidence of an antagonistical reaction was observed by the production of papilla or wall apposition below the contact points of fungus and plant cell walls. Symptomless plants remained symptomless throughout the observation period, and at the ultrastructural level there was no evidence of an antagonistic relationship. This indicates that the symptomless state persists beyond the seedling stage and could contribute, without visual signs, to the total mycotoxin contaminants of corn both before and during kernel development. Thus, studies directed at determining whether the endophytic infection of corn is either latent or biochemically active, and studies on the nature of this apparent compatibility between corn and *F. moniliforme* are warranted.

Fumonisin- and AAL-Toxin-Induced Disruption of Sphingolipid Metabolism with Accumulation of Free Sphingoid Bases[1]

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Fumonisins (FB) and AAL-toxin are sphingoid-like compounds produced by several species of fungi associated with plant diseases. In animal cells, both fumonisins produced by *Fusarium moniliforme* and AAL-toxin produced by *Alternaria alternata* f. sp. *lycopersici* inhibit ceramide synthesis, an early biochemical event in the animal diseases associated with consumption of *F. moniliforme*-contaminated corn. In duckweed (*Lemna pausicostata* Heglem. 6746), tomato plants (*Lycopersicon esculentum* Mill), and tobacco callus (*Nicotiana tabacum* cv Wisconsin), pure FB_1 or AAL-toxin caused a marked elevation of phytosphingosine and sphinganine, sphingoid bases normally present in low concentrations. The relative increases were quite different in the three plant systems. Nonetheless, disruption of sphingolipid metabolism was clearly a common feature in plants exposed to FB_1 or AAL-toxin. Resistant varieties of tomato (*Asc*/*Asc*) were much less sensitive to toxin-induced increases in free sphinganine. Because free sphingoid bases are precursors to plant "ceramides," their accumulation suggests that the primary biochemical lesion is inhibition of de novo ceramide synthesis and reacylation of free sphingoid bases. Thus, in plants the disease symptoms associated with *A. alternata* and *F. moniliforme* infection may be due to disruption of sphingolipid metabolism.

STRATEGIES TO CONTROL FUSARIUM TOXINS IN CORN

TARGETING STRATEGIES FOR CONTROLLING FUSARIA TOXINS IN CORN. I.E. Yates, C.W. Bacon, and D.M. Hinton. R. Russell Agr. Res. Center, ARS, USDA, Athens, GA 30604.

An understanding of the *Fusarium*-corn interaction is essential before meaningful solution(s) can be devised to solve the problem of fusaria toxins in our food and feed supply. This point is underscored by past studies on fescue toxicity where the grass was infected by an endophytic fungus. Much time and many resources were devoted to developing fungus-free fescue because fungus-infected grass caused toxicity in cattle. Unfortunately, the fungal endophyte was discovered to be beneficial to plant growth and development. Thus, fungal free fescue was useless. Another incentive for analyzing the *Fusarium*-corn interaction relates to possible plant growth regulator production by the fungus. A major class of plant growth hormones, gibberellin, was first isolated from plants infected with the sexual form of *Fusarium*, *Gibberella fujkuroi*. Except visible deleterious manifestations, the impact of *Fusarium* on the growth and development of the corn plant has not been characterized. We have observed different patterns of growth in *Fusarium*-infected and non-infected corn plants. Alterations were distinguishable at both the gross morphological and cellular levels. No change in final form was noted, but an acceleration in the rate at which developmental milestones are reached was detected. These manifestations on plant growth and development will be quantified and their relationship and significance to cultural practices and harvestable yield will be explored. Another important aspect of the *Fusarium*-corn interface is identifying factors regulating plant growth that influence the fungal biomass required for toxin accumulation. Molecular markers will be developed to quantify fungal colonization and toxin concentration at various stages of plant growth. Selected *Fusarium* strains will be transformed with the GUS reporter gene so β-glucuronidase activity can be used to quantify and localize the active fungal biomass in corn. Analogous tissues will be collected to quantify fumonisin with fluorescent labeled antibody and immunocytochemical techniques. Corn plants inoculated with *Fusarium* will be grown under controlled greenhouse conditions and treated to varying environmental and nutritional regimes. Fungal growth and toxin concentration will be quantified *in situ* as the corn plant matures. Once the impact of *Fusarium* on growth of the corn plant has been clearly established, a more astute assessment can be made of the level(s) for developing control strategies for toxin production. Such control measures may require an integrated management scheme involving biological, cultural, and chemical means for reducing toxins to acceptable levels. In summary, devising strategies to control fusaria toxins in corn is a complex problem and must be targeted at sites that do not reduce economic crop production.

A BIOCONTROL ENDOPHYTIC BACTERIUM FOR THE ENDOPHYTIC COLONIZATION OF CORN BY *FUSARIUM MONILIFORME*. C. W. Bacon and D.M. Hinton. Toxicology and Mycotoxin Research Unit, USDA, ARS, Athens, GA 30604-5677.

The bacterium *Enterobacter cloacae* has apparent affinities for several grass species, but it is not considered to be an endophyte. This bacterium is presently used for biocontrol of postharvest diseases of fruits and vegetables and as a preplant seed treatment for suppression of damping-off. While screening corn for fungi and bacteria with potential for biocontrol of various corn diseases, surface-sterilized kernels of one unknown Italian corn cultivar produced fungus-free corn seedlings with roots endophytically infected by *E. cloacae*. This paper describes the microscopic nature of *E. cloacae* RRC 101 with corn, and on the *in vitro* control of *Fusarium moniliforme* and other fungi with this bacterium. Light and electron microscopy determined that this isolate of *E. cloacae* was endophytically associated with corn seedling roots, stems and leaves, where it was distributed intercellularly among all tissue except the vascular types. This is a first report of a strain of this bacterium as an endophytic symbiont of plants. It was demonstrated that following a topical application of *E. cloacae* to kernels, and upon germination this bacterium readily infected roots of two other corn cultivars. The bacterium was observed within the endosperm of germinating corn seedling, but germination was not affected. Further, the bacterium was isolated from leaves and stems of 3- to 6-week-old seedlings indicating that the above ground portions of corn were colonized as plant growth proceeded. There was no evidence of damage to cells of the plants during a six to eight week observation period. This bacterium was antagonistic in Petri dish culture to several isolates of the corn pathogen *Fusarium moniliforme*, and to 13 other species of fungi, many of which were corn pathogens. The germination of kernels removed from samples associated with equine leucoencephalomalacia and contained a high fumonisin B1 content was significantly increased in the presence of this bacterium. *E. cloacae* was antagonistic to the growth of *F. moniliforme* in two soil types as evidenced by a 40-fold reduction in colony forming units of the fungus in soils containing the bacterium.

A GENETIC ENGINEERING APPROACH TO LOWERING FUMONISIN LEVELS IN MAIZE KERNELS

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The presence of *Fusarium moniliforme* in asymptomatic, field-grown maize kernels is widespread, if not ubiquitous. Hyphae of *F. moniliforme* in asymptomatic kernels are confined to the pedicel - the basal maternal kernel tissues - where, under advantageous environmental conditions, they can invade the endosperm and embryo, producing fumonisin and other mycotoxins. The pedicel also acts as a conduit for the spread of *F. moniliforme* and other pathogenic fungi to surrounding kernels, via the cob. For these reasons, our approach to lowering mycotoxin levels in maize kernels is to introduce antifungal proteins into the pedicel region of the kernel. Previously, we have isolated and characterized a glutamine synthetase isoform (GSp1) that is found predominately within the pedicel and whose expression is developmentally regulated to correlate with grain fill. Currently, we are isolating the corresponding cDNA from a library prepared from pedicels harvested 15 to 18 days after pollination. Unique 3' or 5' non-coding regions of these clones will be used as probes to isolate the corresponding GSp1 gene from a maize genomic library. The 5' upstream regions of these clones will be analyzed in a seed transient expression system for their ability to express reporter genes within the pedicel region. Suitable promoters will then be fused to the coding regions of antifungal proteins and introduced into maize via microprojectile bombardment.

FUNGAL GENETIC AND MOLECULAR APPROACHES TO FUSARIUM MYCOTOXIN RESEARCH

MEASUREMENT OF FUMONISIN LEVELS IN INDIVIDUAL CORN EARS FROM A TWO YEAR FIELD STUDY

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Fumonisins are produced by *Fusarium moniliforme* and closely related species on corn world wide. Our multi disciplinary research team has identified isolates of *F. moniliforme* that vary in their ability to produce fumonisins in the laboratory. In a two year field study seeds were treated to kill any fungus and then planted as controls or after addition of specific strains of *F. moniliforme*. Plants were samples at various points during growth and the ears were examined by a method utilizing vegetative compatability to determine if they were infected with the strain that was put on the seed, a different strain or no *F. moniliforme*. The strain placed on the seed was recovered in approximately 30% of the analyzed ears. These ears were also examined for fumonisin levels. The field test was conducted at the Rocky Ford experimental plot in Manhattan Kansas. 1993 was the wettest year on record at the test site and the crop had poor yield and severe disease problems. Fumonisin levels overall were quite high in all ears, but visual separation of discolored and damaged kernels was remarkably effective in lowering the overall fumonisin levels in the resulting sound corn. In the 1994 crop the yield was much better and overall disease levels were lower. Fumonisin levels overall were much lower and fumonisins were undetectable in many ears. Again visually separated discolored and damaged kernels had significantly higher fumonisin concentrations. The overall fumonisin levels in the sound fractions were not statistically different in 1993 and 1994 corn.

BIOCHEMISTRY AND GENETICS OF FUMONISIN BIOSYNTHESIS

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Fumonisin mycotoxins are produced by *Gibberella fujikuroi* (*Fusarium moniliforme*) mating population A, a major pathogen of maize and sorghum worldwide. Our multidisciplinary research team is identifying fumonisin biosynthetic genes by both classical meiotic and molecular genetic methods. Meiotic analysis of naturally occurring fumonisin production mutants has identified three putative fumonisin biosynthetic loci. *Fum1*, which can control the ability to produce fumonisins, has been localized by marker-based mapping to one end of chromosome 1. *Fum2* and *fum3* appear to control hydroxylation of carbon-10 and carbon-5, respectively. Allelism tests indicate that *fum1*, *fum2* and *fum3* are tightly linked and may constitute a gene cluster on chromosome 1. This finding is not unexpected because genes that control biosynthesis of aflatoxins and trichothecenes in several fungal species have recently been found to be organized in complex clusters. When marker-based mapping has localized the position of the fumonisin biosynthetic genes to a particular chromosomal region or regions, the genes will be identified by complementation of function via DNA-mediated transformation. Understanding fumonisin biosynthesis and its genetic regulation should facilitate development of measures to control fumonisin contamination.

SIGNIFICANCE OF MOLECULAR APPROACHES TO *FUSARIUM* MYCOTOXIN RESEARCH

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Recent advances in the area of mycotoxin biosynthesis research have created opportunities for the application of direct molecular approaches to the isolation of mycotoxin pathway genes. The clustering of mycotoxin pathway genes has been observed for both trichothecene and aflatoxin pathways suggesting that gene clustering is a general feature of mycotoxin pathway gene organization. This is because gene clusters facilitate efforts to isolate pathway genes by presenting larger targets for molecular gene isolation strategies. It has also been observed that the branch point steps in both polyketide and non-ribosomal peptide pathways employ enzymes that contain highly conserved sequences useful for the design of molecular probes. Exploiting these aspects of mycotoxin pathways will greatly speed-up efforts to characterize pathway genes. We are presently employing these approaches to identify genes for the polyketide mycotoxins, fumonisin and zearalenone, and for the non-ribosomal peptide mycotoxin, beauvericin. Isolation of genes encoding branch-point step enzymes will permit us to create genetically defined mycotoxin-deficient mutants by gene disruption for studies of mycotoxin involvement in plant disease. These mutants will also be useful determining the contribution of specific mycotoxins to animal toxicity within the complex mixtures of mycotoxins produced by some *Fusarium* species.

TRICHOTHECENE RESEARCH
INCLUDING WHEAT HEAD SCAB

TRICHOTHECENE BIOSYNTHESIS IN *FUSARIUM*

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The pathway for trichothecene toxin biosynthesis in *Fusarium* is being characterized by biochemical and molecular genetic methods. The biosynthesis of all trichothecenes proceeds from the sesquiterpene hydrocarbon trichodiene. The ordered sequence of oxygenations, isomerizations, cyclizations and esterifications leading from trichodiene to the more complex trichothecene toxins such as vomitoxin, diacetoxyscirpenol, and T-2 toxin has been established through experiments with *Fusarium sporotrichioides*, *F. sambucinum, F. graminearum*, and *F. culmorum.* For T-2 toxin biosynthesis: trichodiene → 2-hydroxytrichodiene → 12,13-epoxytrichoene-2-ol → isotrichodiol → isotrichotriol → trichotriol → isotrichodermol → isotrichodermin → 15-decalonectrin → calonectrin → 3,15-diacetoxyscirpenol → 3,4,15-triacetoxyscirpenol → 3-acetylneosolaniol → 3-acetyl T-2 toxin → T-2 toxin. Trichothecene biosynthesis of vomitoxin shares most of the initial steps of this pathway; the branch point appears to occur after 15-decalonectrin. Trichothecene biosynthetic genes are clustered. At least eight genes involved in *F. sporotrichioides* trichothecene biosynthesis have been shown to be present on a single cosmid clone. The functions of these genes have been determined through biochemical analysis of mutants generated by molecular gene disruption. These include a sesquiterpene cyclase, cytochrome P450 monooxygenases, transacetylases and regulatory genes. By using molecular disruption of the gene encoding the sesquiterpene cyclase, mutants of *F. sporotrichioides*, *F. sambucinum,* and *F. graminearum* have been generated. Transformants carrying the disrupted allele produce no trichothecenes.

TRICHOTHECENE TOXINS AND WHEAT HEAD SCAB

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Fusarium graminearum causes head blight (scab) of wheat, a disease that causes severe losses in the wheat crop of the eastern and central United States. In addition to reducing yield, the fungus produces trichothecene mycotoxins, such as deoxynivalenol, that are harmful to animals and humans that consume infected grain. The considerable amount of genetic information and energy devoted to trichothecene production in *Fusarium* suggests these toxins confer some advantage to the fungus. To determine whether trichothecene production contributes to the ability of *F. graminearum* to cause head blight, we generated trichothecene nonproducing mutants of the fungus by transformation mediated gene disruption of the *Tri5* gene. This gene encodes trichodiene synthase, the enzyme that catalyzes the first step in trichothecene biosynthesis. The virulence of two trichothecene nonproducing mutants was examined on wheat (cultivar Wheaton) plants under field conditions. The mutants caused less disease compared to the wild type trichothecene producing strain from which they were derived. To confirm that the reduced virulence of the mutants was due specifically to the disruption of the *Tri5* gene rather than some nonspecific effect of the transformation process, a mutant strain was induced to revert to wild type. In the revertant strain, the disrupted *Tri5* reverted to the wild type *Tri5* and as a result the strain regained the ability to produce trichothecenes. The revertant strain also regained the ability to cause high levels of disease. These results indicate that the trichothecene production contributes to the ability of *F. graminearum* to cause head blight of wheat and suggest that trichothecene resistant wheat may offer a means to control head blight and the resulting mycotoxin contamination problem.

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